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14. ABSTRACT

We proposed that alterations in histone methylation regulate MSC fate commitment and predispose these progeny to malignant transformation. Transformed ER+ epithelial cells deregulate proliferation of MSC and luminal progenitors contributing to transformation of ER- luminal and basal cells and development of treatment resistant breast cancer. Specific Aim #1 tested the hypothesis that EZH2 regulates both ER and DNA repair gene expression in MSC resulting in the differentiation of ER+ but transformation sensitive mammary epithelial cells. Our preliminary results indicate that inhibiting EZH2 expression in luminal progenitor cells significantly reduced histone H3K27me3 levels. These epigenetic changes increased DNA repair activity in luminal progenitor cells. DNA repair was significantly inhibited in MSC transduced with Rad50 and NBS1 shRNAs. Transplanted MSC with reduced Rad50 or NBS1 expression but not EZH2 inhibited luminal progenitors reconstituted mammary fat pads.

15. SUBJECT TERMS
histone methylation, mammary stem cell, estrogen receptor, luminal progenitor, DNA repair

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INTRODUCTION

Breast cancer and its treatment impose significant physical, psychological, and economic burdens on patients, their families, and society. African-American women under age 35 have the highest age adjusted mortality rates from breast cancer. The incidence of breast cancer in this group also is higher than for Caucasian women. In several studies these disparities persisted after other variables such as socioeconomic status and access to care were controlled. These studies point to biological differences in breast cancer among these populations such as increased prevalence of the high grade, estrogen receptor negative, basal subtype of the disease in African-American women. Targeting estrogen receptor α (ER) action is a classic example of molecular therapy for breast cancer (for review see Russo et al., 2000; Crowe, 2002). ER- mammary stem cells (MSC) are believed to be the progenitor population for all breast epithelia including acinar cells and the largely ER- basal and luminal cells of the ducts from which most breast cancer arises (for review see Crowe et al., 2004; Visvader and Lindeman, 2006; Sleeman et al., 2007). The luminal epithelial population also contains a subpopulation of ER- progenitors (Asselin-Labat et al., 2007). ER is expressed by a fraction of luminal epithelial cells in normal mammary glands, but the numbers of these cells often are greatly increased in human breast cancer. MSC can reconstitute mammary glands and have been isolated from both humans and mice using cell surface markers (Gudjonsson et al., 2002; Dontu et al., 2003; Stingl et al., 2006; Shackleton et al., 2006). Some of these MSC populations are expanded in mouse mammary cancer models (Li et al., 2003; Liu et al., 2004), and tumorigenic progenitor populations have been isolated from human breast cancers (Al-Hajj et al., 2003; Ponti et al., 2005). These studies have demonstrated an important role for MSC in mammary gland development and tumorigenesis. However these models have not determined how MSC or luminal progenitors give rise to ER+ epithelial cells during mammary gland development, what predisposes ER+ cells to malignant transformation, and how these cells regulate breast tumorigenesis in conjunction with transformed ER- MSC and luminal progenitors. In clinical studies, EZH2 histone methyltransferase (HMT) expression has been associated with poorly differentiated and aggressive breast cancer in humans (Kleer et al., 2003; Raaphorst et al., 2003; Bachmann et al., 2006; Collett et al., 2006; Ding et al., 2006). The histone demethylase JMJD3 reverses the EZH2 mediated histone H3 lysine-27 methyl mark. We proposed a new mechanism of breast tumorigenesis that, during normal mammary gland development, decreased expression of DNA repair genes during HMT mediated differentiation of MSC to ER+ luminal cells makes the latter population more susceptible to transformation and expansion. These transformed cells may induce aberrant proliferation of the ER- MSC or luminal progenitors resulting in genomic instability, production of additional transformed ER+ or ER- luminal cells, or transformation of MSC giving rise to the aggressive basal subtype of mammary cancer. We hypothesized that alterations in histone methylation regulate MSC fate commitment and predispose these progeny to malignant transformation. Transformed ER+ epithelial cells deregulate proliferation of MSC and luminal progenitors contributing to transformation of ER- luminal and basal cells and development of treatment resistant breast cancer. **Specific Aim #1** will test the hypothesis that EZH2 regulates both ER and DNA repair gene expression in MSC resulting in the differentiation of ER+ but transformation sensitive mammary epithelial cells. Specific Aim #1 will test this hypothesis by comparing transformation of these cell populations following altered histone methylation. **Specific Aim #2** will test the hypothesis that transformed ER+ cells regulate proliferation and tumorigenicity of the MSC and luminal progenitor populations by determining the molecular and cellular effects of co-transplantation of these populations. Specific Aim #2 also will target histone and DNA methylation in these cells using molecular therapy to determine the effects on mammary tumorigenesis. Understanding the relationships between normal and transformed mammary epithelial cells has important ramifications for preventing ER- and basal subtype breast cancer in African-American women. The biologically aggressive basal subtype of breast cancer may result from transformation of the less differentiated MSC population. Targeting interactions between ER+ cells and MSC to prevent progression to these treatment resistant tumors will improve survival and quality of life.

BODY

The data obtained during year 1 of the award are preliminary and subject to modification as additional data are obtained. We sorted mammary epithelial stem cells and luminal progenitors from mouse mammary fat pads using established markers CD24+ CD49f++ and CD24+ CD49f- CD61+ respectively (Fig. 1). We obtained 0.1% CD24+ CD49f++ MSC and 5.1% CD24+ CD49f- CD61+ luminal progenitor populations. We inhibited EZH2 expression in luminal progenitors, and Rad50 and NBS1 expression in MSC by transduction of shRNAs. EZH2, Rad50, and NBS1 expression was inhibited by 80-90% as determined by qRT-PCR (Fig. 2). Histone H3K27me3 levels of the ER, Rad50, and NBS1 promoters were determined in luminal progenitors using chromatin immunoprecipitation, and expression of these genes was determined by qRT-PCR. As shown in Fig. 3A, histone H3K27me3 levels at the indicated regulatory regions were reduced in EZH2-inhibited luminal progenitors by up to 60%. Reduced H3K27me3 levels correlated with 2-3 fold increased expression of ER, Rad50, and NBS1 mRNA (Fig. 3B). EZH2-inhibited luminal progenitors exhibited an 86% increase in DNA repair activity by plasmid end joining analysis (Fig. 3C). DNA repair was significantly inhibited (75-80%) in MSC transduced with Rad50 and NBS1 shRNAs in this assay.

We transplanted luminal progenitors with reduced EZH2 expression, MSC with reduced Rad50 and NBS1 expression, and control clones into cleared mammary fat pads. Reconstitution of cleared mammary fat pads by EZH2 inhibited luminal progenitors was reduced by 80% compared to transplanted MSC as determined by terminal end bud quantitation (Fig. 4). We transformed EZH2-inhibited luminal progenitors and MSC with decreased Rad50 and NBS1 expression using a standard protocol (Hahn et al., 1999). These cells have been transplanted to cleared mammary fat pads. At the time of this writing, control clones have formed tumors in recipient mouse mammary fat pads (Fig. 5). We are monitoring tumor formation in cleared mouse mammary fat pads transplanted with EZH2-inhibited luminal progenitor cells and MSC in which Rad50 or NBS1 expression was reduced by shRNA transduction. The increased tumor latency of these cells was expected due to activation of programmed cell death mechanisms. In the funded application we proposed monitoring these transplanted fat pads for up to 1 year. We should have final histopathologic, immunofluorescent cellular localization, and flow cytometric analyses of these tumors completed within a few months.

KEY RESEARCH ACCOMPLISHMENTS

- Using shRNA technology, we inhibited EZH2, Rad50, and NBS1 expression by 80-90% in MSC and luminal progenitors.
- Histone H3K27me3 levels were reduced in EZH2-inhibited luminal progenitors by up to 60%.
- EZH2-inhibited luminal progenitors exhibited increased DNA repair activity.
- DNA repair was significantly inhibited in MSC transduced with Rad50 and NBS1 shRNAs.
- Transplanted MSC with reduced Rad50 or NBS1 expression reconstituted duct formation in cleared mammary fat pads.
- Transplanted luminal progenitor cells with inhibited EZH2 expression exhibited minimal reconstitution of cleared mammary fat pads.
- Transformed control MSC formed tumors in cleared mammary fat pads.

REPORTABLE OUTCOMES

We have made excellent progress in year 1 toward accomplishing the specific aims outlined in the funded application. We have not yet submitted an abstract or manuscript detailing the conclusions of the project.

CONCLUSION

We have completed the proposed work in Specific Aim 1 of the funded application and are currently evaluating tumor formation resulting from genetically altered transplanted MSC and luminal progenitor cells. Our

preliminary results indicate that inhibiting EZH2 expression in luminal progenitor cells significantly reduced histone H3K27me3 levels. These epigenetic changes increased DNA repair activity in luminal progenitor cells. DNA repair was significantly inhibited in MSC transduced with Rad50 and NBS1 shRNAs. Transplanted MSC with reduced Rad50 or NBS1 expression reconstituted cleared mammary fat pads. Luminal progenitors with inhibited EZH2 expression minimally reconstituted mammary fat pads. We are currently assessing tumor formation in cleared mammary fat pads following transformation of genetically altered MSC and luminal progenitors. These results have important implications for mammary gland development and cellular transformation. These studies will determine a mechanism for how MSC and luminal progenitor cells give rise to ER+ luminal cells during mammary gland development, how these progenitor populations are prone to malignant transformation, and ultimately how ER+ cells regulate breast tumorigenesis in conjunction with transformed ER- MSC and luminal progenitor cells.

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APPENDICES

Not applicable

SUPPORTING DATA

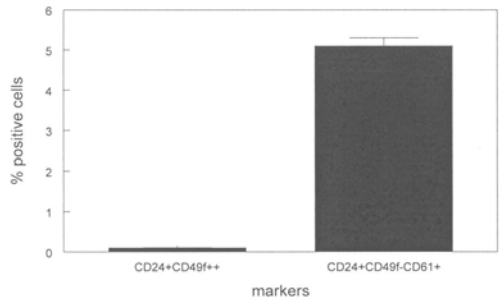

Fig. 1. Percentage of CD24+CD49f++ mammary stem and CD24+CD49f-CD61+ luminal progenitor cells sorted from normal mouse mammary gland.

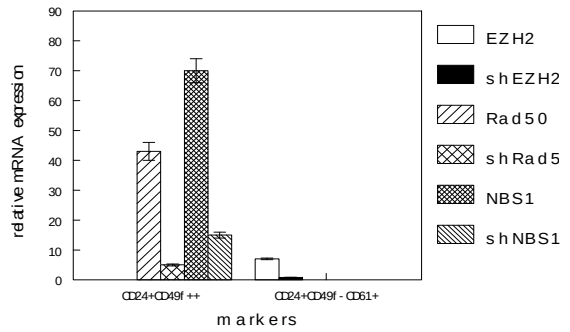

Fig. 2. Inhibition of EZH2 expression in luminal progenitor cells (CD24+CD49f-CD61+) and Rad50 and NBS1 levels in CD24+CD49f++ mammary stem cells by shRNA transduction. Gene expression was determined by qRT-PCR. Error bars indicate SEM.

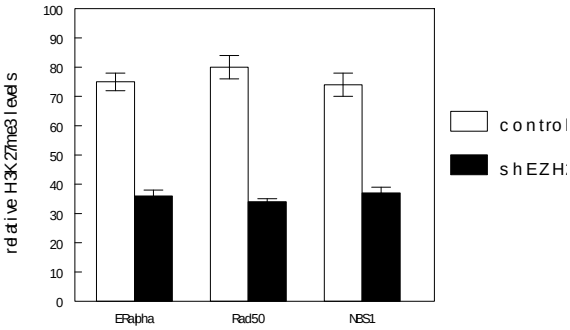

Fig. 3A. Reduced histone H3K27me3 levels at ERalpha, Rad50, and NBS1 regulatory regions in luminal progenitor cells with decreased EZH2 expression (shEZH) compared to control cells. H3K27me3 occupancy was determined by chromatin immunoprecipitation. Error bars indicate SEM.

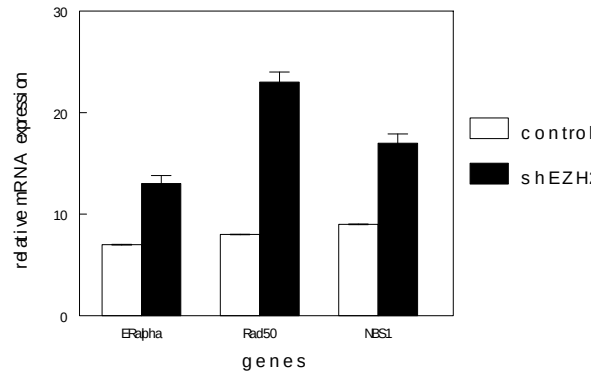

Fig. 3B. EZH2 inhibition (shEZH) increases ERalpha, Rad50, and NBS1 expression in luminal progenitor cells compared to control cells. Error bars indicate SEM.

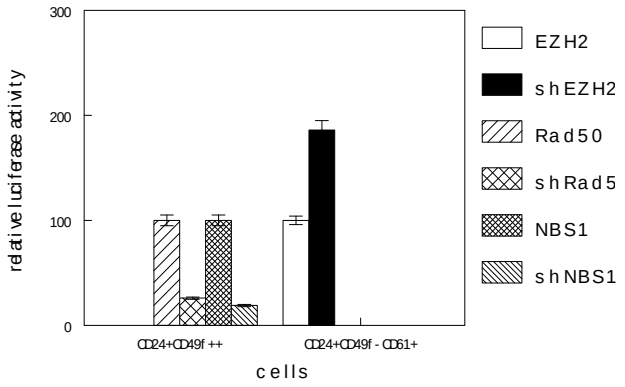

Fig. 3C. Rad50 and NBS1 inhibition (shRad, shNBS1) reduces DNA repair activity in CD24+CD49f++ MSC cells. EZH2 inhibition (shEZH2) increases DNA repair activity in CD24+CD49f-CD61+ luminal progenitor cells. DNA repair activity was measured by reconstitution of luciferase activity in plasmid end joining analysis. Error bars indicate SEM.

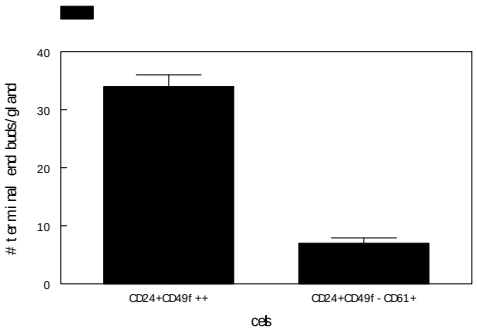

Fig. 4. Reduced mammary gland reconstitution in CD24+CD49f-CD61+ luminal progenitors compared to CD24+CD49f++ MSC. The number of terminal end buds in each mammary fat pad was counted following whole mount staining. Error bars indicate SEM.

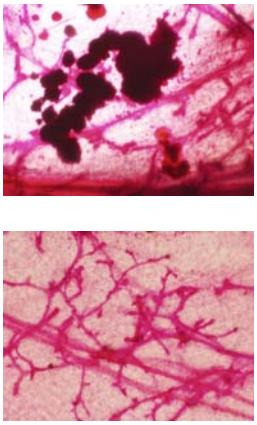

Fig. 5. Early mammary tumor formation in transformed control MSC (upper panel) compared to wild type mammary gland (lower panel) is shown by whole mount mammary gland staining. Representative photomicrographs are shown.